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Award Number: DAMD17-00-1-0145

TITLE: Mechanism of Mutation in Non-Dividing Cells

PRINCIPAL INVESTIGATOR: Rebecca G. Ponder
Susan Rosenberg

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary (1 Jul 2000 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Mechanism of Mutation in Non-Dividing Cells

5. FUNDING NUMBERS
DAMD17-00-1-0145

6. AUTHOR(S)
Rebecca G. Ponder
Susan Rosenberg

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail: rp692236@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Stationary-phase mutation can be induced in non-dividing cells after exposure to environmental stress. The mutations require the SOS-induced, error-prone polymerase DAN pol IV and DNA double-strand break (DSB) repair and recombination proteins. Sex plasmid transfer proteins are required for mutation of lac on the F'; transfer functions induce single-strand nicks that might become DSBs. We find that introducing specific breaks in cis to lac in the absence of transfer functions results in ~1000-fold stimulation of Lac+ stationary-phase mutation, including both frameshift reversions and lac gene amplifications. The mutation requires recombination proteins and pol IV, implying that these components act downstream of DSBs. DSBs made in trans to lac promote Lac+ mutation only poorly. We suggest adaptive mutations result form error-prone DNA synthesis primed during recombination- mediated DSB-repair, and the small stimulation provided by DSBs in trans results form SOS induction and increased pol IV. Notabl, we report that DSBs made in trans can promote Lac+ mutation if a region of DNA homology with the trans plasmid is provided near lac. Finally, we find that DSBs are not sufficient to activate mutation: DSB-activated mutaton has not been observed in rapidly growing cells and requires RpoS, the stationary-phase and general stress response regulator.

14. SUBJECT TERMS
breast cancer, mutation, recombination, error-prone polymerase double-strand break

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

Stationary-phase mutation, or adaptive mutation, refers to a collection of mutagenic responses that can be induced in stationary-phase (non-growing) cells after exposure to environmental stress. In the *E. coli* Lac system, cells carrying a chromosomal *lac* deletion and an F' sex plasmid with a *lac* +1 frameshift allele generate Lac^+ reversion mutants over time when starved on medium with lactose as the only carbon source. The mechanism for stationary-phase mutation is intrinsically different from that of growth-dependent mutation; it requires the homologous recombination proteins RecA, RecBCD, and RuvA, RuvB, and RuvC. RecA is a homolog of the human protein RAD51, which associates with the DNA repair BRCA tumor suppressor proteins. RecBCD is the major double strand break (DSB) repair enzyme in *E. coli.* The SOS-inducible, error-prone DNA polymerase, pol IV (or DinB) is also required for stationary-phase Lac^+ reversion; this enzyme is a homolog of four new human DNA polymerases: RAD30a (the XPV tumor suppressor protein), RAD30b, REV1, and DINB1. The mechanisms by which these proteins act in environmentally-inducible mutation are likely relevant to cancer formation, tumor progression, and chemotherapeutic drug resistance in humans.

DNA DSBs have been implicated as molecular intermediates to stationary-phase mutation because of the requirement for RecBCD; the enzyme loads only onto DNA ends. In one model, recombination-mediated repair of a DSB is suggested to promote mutation by priming DNA replication using DNA pol IV, during which polymerase errors occur. Cells carrying mutations that revert the *lac* +1 frameshift are able to utilize lactose in the medium and grow, escaping stress. Although stationary-phase mutation appears to occur throughout the genome, on the bacterial chromosome as well as the F' sex plasmid, the frequency of mutation varies widely from locus to locus. For example, the F' *lac* +1 framshift normally used in our assays mutates at a frequency of about $1x10^{-6}$ mutants per cell over the course of five days, whereas the frequency of mutation of a frameshift at the chromosomal *lac* locus is less than $1x10^{-8}$. We hypothesize that DSBs activate mutation in stationary-phase, and the rate of recombination-dependent mutation at a locus is directly affected by its proximity to DSBs.

DSBs could arise naturally in cells from DNA synthesis across an existing single-stranded nick, an induced enzymatic activity in stationary-phase, or an increased rate of oxidative damage (and its processing by endonucleases during repair). In the case of the F', we know that plasmid-encoded transfer (Tra) proteins are required for stationary-phase mutation, although actual conjugative transfer is not. An endonuclease called TraI induces single-strand nicks at the origin of transfer on the F', and there are many ways in which a nick might become a DSB, such as a nick on the opposing DNA strand or passage of a replication fork. We hypothesize that Tra proteins activate mutation on the F' because they promote DSBs by providing single-strand nicks. The goal of this project is to determine the role of DNA DSBs and DSB repair in Lac^+ recombination-dependent stationary-phase mutation in *E. coli.*

Body:

We have asked whether DSBs introduced specifically near *lac* on the F' can 1) activate stationary-phase mutation and 2) substitute for Tra functions. To make specific DSBs, I constructed strains that express the *S. cerevisiae* endonuclease I-SceI (substituted for yeast HO endonuclease, as described in SOW Task 1(b), months 1-5) under the arabinose promoter, P_{BAD}, from *attB* in the *E. coli* chromosome. At the same time, I cloned the I-SceI restriction site, an 18bp sequence not present in the *E. coli* genome, into a defective miniTn7, and moved the miniTn7 into multiple sites to the left and to the right of the +1 *lac* frameshift mutation on the F' sex plasmid. Once the desired cut sites were identified, I constructed strains that carry the specific I-SceI restriction sites on an F' deleted for TraI endonuclease and either the P_{BAD}-I-SceI gene or P_{BAD} alone at *attB* in the *E. coli* chromsome.

P_{BAD} is induced by arabinose and repressed by glucose or fucose, so we can control expression of I-SceI in our strains. However, if we plate the cells on arabinose and induce DSBs, death is observed (only) in strains carrying both the I-SceI gene and a cut site on the F'. In the stationary-phase mutation assays I am doing, cultures of the strains to be tested are grown in minimal glycerol medium with 0.001% glucose added for repression of the arabinose promoter. Cultures are washed twice and plated on minimal lactose plates without arabinose, so any I-SceI endonuclease produced is a result of leaky expression from P_{BAD} in the absence of inducer and repressor. The number of Lac^+ colonies are then counted daily until five days after plating. Under these conditions, we know DSBs are made because strains carrying both the I-SceI gene and a cut site still exhibit some death, such that the number of viable cells drops three- to five-fold over the course of five days.

In repeated sets of experiments (SOW Task 1(b), months 10-15), introduction of specific DSBs at cut sites to the left and to the right of the *lac* +1 frameshift allele on a Tra-defective F' caused dramatic 1000-fold stimulations of Lac^+ stationary-phase mutation. (figures 1 and 2). This effect was DSB-dependent because no increase in mutation was seen in any of the controls with enzyme but no cut site or cut site but no enzyme. These results provide the first direct evidence that DSBs can activate stationary-phase mutation. Because the DSBs substitute for TraI single-strand endonuclease, the results also imply that the role of Tra functions in adaptive mutation is to promote DSBs (rather than for F' transfer or transfer replication).

Under normal conditions, the majority of stationary-phase Lac^+ isolates carry mutations that restore the *lac* reading frame, but a small proportion of Lac^+ colonies result from adaptive amplification. These cells have become phenotypically Lac^+ by acquiring several tandem copies of the leaky *lac* +1 frameshift allele, and can be distinguished from Lac^+ point mutants because they show characteristic blue/white sectoring on nonselective (rich) X-gal medium. Much less is know about adaptive amplification than stationary-phase point mutation, but we do know that amplification does not require DNA pol IV. To characterize the DSB-activated mutants, I streaked up to 42 DSB-stimulated Lac^+ isolates per culture per day of the experiment to X-gal medium and found that introducing specific DSBs near *lac* activates adaptive amplification, although the majority of Lac^+ revertants result from point mutation (figure

3). In addition, the proportion of amplified colonies out of the total Lac^+ is smaller in the DSB-stimulated background than in a Tra^+ control strain.

We have also asked whether the introduction of DSBs activates a similar mechanism to that which produces Lac^+ stationary-phase mutation, requiring recombination proteins and DNA pol IV, or an alternative pathway. In repeated sets of experiments, loss of any of the recombination proteins RecA, RecB, and RuvC resulted in a dramatic decrease of the DSB-stimulated mutation (figure 4). Similarly, the break-promoted mutation required DNA pol IV (figure 5). These results indicate, first, that introduced DSBs near *lac* activate a mutation mechanism(s) similar to those stationary-phase mechanisms normally observed in the Lac system. Second, these data indicate that the functions of RecA, RecBCD, Ruv proteins, and DinB/Pol IV in stationary-phase mutation are required after DSB formation (DSBs can not substitute for them). This result rules out previously plausible models in which these proteins act solely in generation of DSBs and supports models in which stationary-phase mutation is directly associated with DSB repair.

Since my last report, we have asked whether the introduction of DSBs can substitute for stationary-phase in the pathways leading to F' Lac^+ mutation; that is, whether DSBs can activate recombination-dependent, DNA pol IV-dependent mutation in growing cells. Arabinose was added to culture medium to induce I-SceI and make specific DSBs near a *tet* +1 frameshift target allele on a Tra-defective F'; mutants were selected on the antibiotic tetracycline. We know that DSBs were made during growth because those strains that carried both cut site and enzyme had slower growth curves and took longer to saturate than strains with cut site only. In repeated sets of experiments, introduction of specific DSBs caused two-log stimulations of pol IV-dependent mutation to TetR on a Tra-defective F' in culture, but only in stationary-phase, not log phase, cells (figure 6). If true, this result would indicate that there is some other component that is provided in stationary-phase cells that is necessary for the mutational mechanim(s) to activate. Unfortunately, it is hard to interpret this negative result because it is possible that TetR mutants did arise during growth in log phase, but we were unable to rescue them, or that the ratio of cut to uncut molecules (from which to repair) was different.

As a second, indirect way to ask whether DSBs are sufficient to activate stationary-phase mutation, we asked whether DSBs can overcome the requirement for the stationary-phase and general stress response sigma (transcription) factor of RNA polymerase, RpoS. Loss of RpoS causes an approximate ten-fold decrease in stationary-phase mutation in a wild-type background. Similarly, in repeated sets of experiments, I have seen that the majority of the DSB-activated mutation is RpoS-dependent (figure 7). This result, in conjunction with our inability to detect DSB-activated mutation in growing cells, supports the hypothesis that DSBs can not substitute for stationary-phase.

We asked whether DSBs activate mutation only *in cis* or also *in trans*. In a "*cis*" model for stationary-phase mutation, recombination-mediated repair of a DSB primes error-prone DNA synthesis using DNA pol IV at *lac*. In this model, the recombinational repair of the DSB and resulting mutation occur *in cis* on the DNA. However, we can also draw a "*trans*" model in which a DSB leads to induction of the SOS response, pol IV upregulation, and polymerase errors in areas of DNA synthesis throughout the cell, *in trans* to the DSB repair. I have already shown that specific DSBs in *cis* to *lac* can activate stationary-phase mutation on a Tra-defective F' (figures 1 and 2). To test whether DSBs made in *trans* would also activate mutation, I created strains that carry either the P_{BAD}-I-SceI gene or P_{BAD} alone at *attB* and an I-SceI cut site at *upp* in the chromosome. In repeated sets of experiments, introduction of specific DSBs at *upp*, in *trans* to the *lac* +1 frameshift on a Tra-defective F', had no effect on Lac^+ stationary-phase mutation. Unfortunately, we could not conclude from this result that DSBs activate stationary-phase mutation by a *cis* mechanism because we could not show that similar numbers of DSBs were created at the various cut sites on the F' and the chromosome. We also could not control for the possibility that introducing DSBs in the chromosome was more lethal to a cell than making breaks on the F'.

To overcome these difficulties, we decided to assay the effect of DSBs made on a third replicon, a pBR322-based plasmid. In this case, making DSBs would not affect cell viability. In repeated sets of experiments, introduction of specific DSBs on an unselected plasmid, *in trans* to *lac*, caused only small (three to six-fold) stimulations of Lac^+ stationary-phase mutation on a Tra-defective F' (figure 8). This level of activation is at least 100-fold less than when DSBs are provided *in cis*. We conclude that stationary-phase mutation occurs mostly by a *cis*, rather than a *trans*, mechanism.

The *cis* model for stationary-phase mutation described above predicts that DNA ends created from a DSB on one molecule can provoke mutation on a second, uncut molecule during homology-mediated repair. I have shown that specific DSBs introduced on a plasmid *in trans* to *lac* activate stationary-phase mutation only slightly when no homology exists between the two molecules. I asked whether the addition of DNA homologous to the plasmid cut end to the F' could further stimulate DSB-activated stationary-phase mutation on the uncut, Tra-defective F' (similar to experiments outlined in Task 2, but not quite the same). In repeated sets of experiments, introduction of specific DSBs on the *trans* plasmid in the presence of F' homology caused ten-fold greater stimulations of Lac^+ stationary-phase mutation than DSBs *in trans* alone (figure 9). This further supports models in which homologous interaction between a DNA end and DNA near *lac* promotes mutation. However, one caveat to this result is the possibility that the plasmid containing the I-SceI site integrated permanently or transiently into the F', such that DSBs were actually provided in *cis* to *lac*. These events should be rare, but I am currently screening a number of *trans* plasmid plus homology Lac^+ isolates for the presence of plasmid DNA around the site of homology on the F'. I know already that 27 out of 32 F's examined do not carry a co-integrated plasmid. We may in the future assay for direct exchange of markers between the plasmid and F' to ask whether recombined DNA is linked to DNA that has mutated.

All of the work described thus far has studied activation of stationary-phase mutation on a Tra-defective F'. I have also constructed a similar set of I-SceI strains to ask whether introduction of specific DSBs can activate reversion of a +1 frameshift at the chromosomal *lac* locus, a site notoriously cold for stationary-phase mutation. Experiments using these strains have been placed on hold. Please be aware that none of this material has been published, with the exception of the P_{BAD}-I-SceI allele construction.

Key Research Accomplishments (July 2000-July 2003):

- Gathered the first direct evidence that DSBs activate Lac$^+$ stationary-phase mutation
- Demonstrated that DSBs can substitue for transfer functions in stationary-phase mutation
- Showed that DSBs activate both stationary-phase point mutation and adaptive amplification
- Showed that the DSB-stimulated mutation requires recombination proteins and DNA pol IV
- Gathered evidence that DSBs alone are not sufficient to activate stationary-phase mutation
- Demonstrated that DSBs activate stationary-phase mutation by a *cis*, rather than a *trans*, mechanism
- Showed that homologous interactions promote stationary-phase mutation
- Mentored 5 students in projects dealing with mutation and recombination in *E. coli.*

Reportable Outcomes:

Publications:

L. M. Gumbiner-Russo et al. 2001. The TGV *T*ransgenic *V*ectors for Single Copy Gene Expression from the *Escherichia coli* Chromosome. *Gene* 273: 97-104.

R. G. Ponder and S. M. Rosenberg. A direct role for DNA double-strand breaks and their repair in stationary-phase mutation (in preparation for *Cell*).

Presentations:

August 2001. R. G. Ponder, N. L. Craig, and S. M. Rosenberg. "Direct Evidence for Double-Strand Breaks in Stationary-Phase Mutation." The 2001 Molecular Genetics of Bacteria and Phages Meeting at University of Wisconsin-Madison. Oral presentation.

August 2002. R. G. Ponder and S. M. Rosenberg. "Double-Strand Break-Activated Stationary-Phase Mutation Requires Rec Proteins and DNA PolIV/ DinB." The 2002 Molecular Genetics of Bacteria and Phages Meeting at Cold Spring Harbor, NY. Poster presentation.

September 2002. R. G. Ponder and S. M. Rosenberg. "Role of DNA Double-Strand Breaks in Mutation in Growth-Inhibited Cells." The Era of Hope Department of Defense Breast Cancer Research Program Meeting in Orlando, FL. Poster presentation.

October 2002. R. G. Ponder and S. M. Rosenberg. "A Direct Role for DNA Double-Strand Breaks and Their Repair in Stationary-Phase Mutation." The University of Texas M. D. Anderson Cancer Center 55th Annual Symposium on Fundamental Cancer Research: Maintenance of Genomic Integrity in Houston, TX. Poster presentation.

Conclusions:

The mechanism for stationary-phase mutation requires the homologous recombination proteins RecA, RecBCD, and RuvABC and the SOS-inducible, error-prone DNA polymerase, polIV. Some of these prokaryotic DNA repair and mutation proteins are homologs of human DNA damage response proteins; RecA is a homolog of hRAD51, which associates with the DNA repair BRCA tumor suppressor proteins, and *E. coli* DNA polIV, or DinB, is a homolog of four new human DNA polymerases: RAD30a (the XPV tumor suppressor protein), RAD30b, REV1, and DINB1. The mechanisms by which these proteins act in environmentally-inducible mutation are likely relevant to cancer formation, progression, and resistance to chemotherapeutic drugs in humans.

We find that introducing specific breaks at sites on either side of *lac* on a transfer-defective F' causes 1000-fold stimulations of *E. coli* Lac$^+$ stationary-phase mutation. The data imply that the role of Tra functions in stationary-phase mutation is to make DSBs by providing single-strand nicks, and provide direct evidence that DSBs can activate stationary-phase mutation in the Lac system. This activation of mutation includes both point mutation and adaptive gene amplification and requires recombination proteins and DNA pol IV, indicating that these proteins work downstream of DSBs in pathways leading to mutation. DSBs promote mutation directly, *in cis*, by homologous interaction with the DNA molecule that gets mutated, but only in the context of stationary-phase, RpoS regulon-expression.

Figure 1

DSBs to the left of *lac* ↑↑ mutation

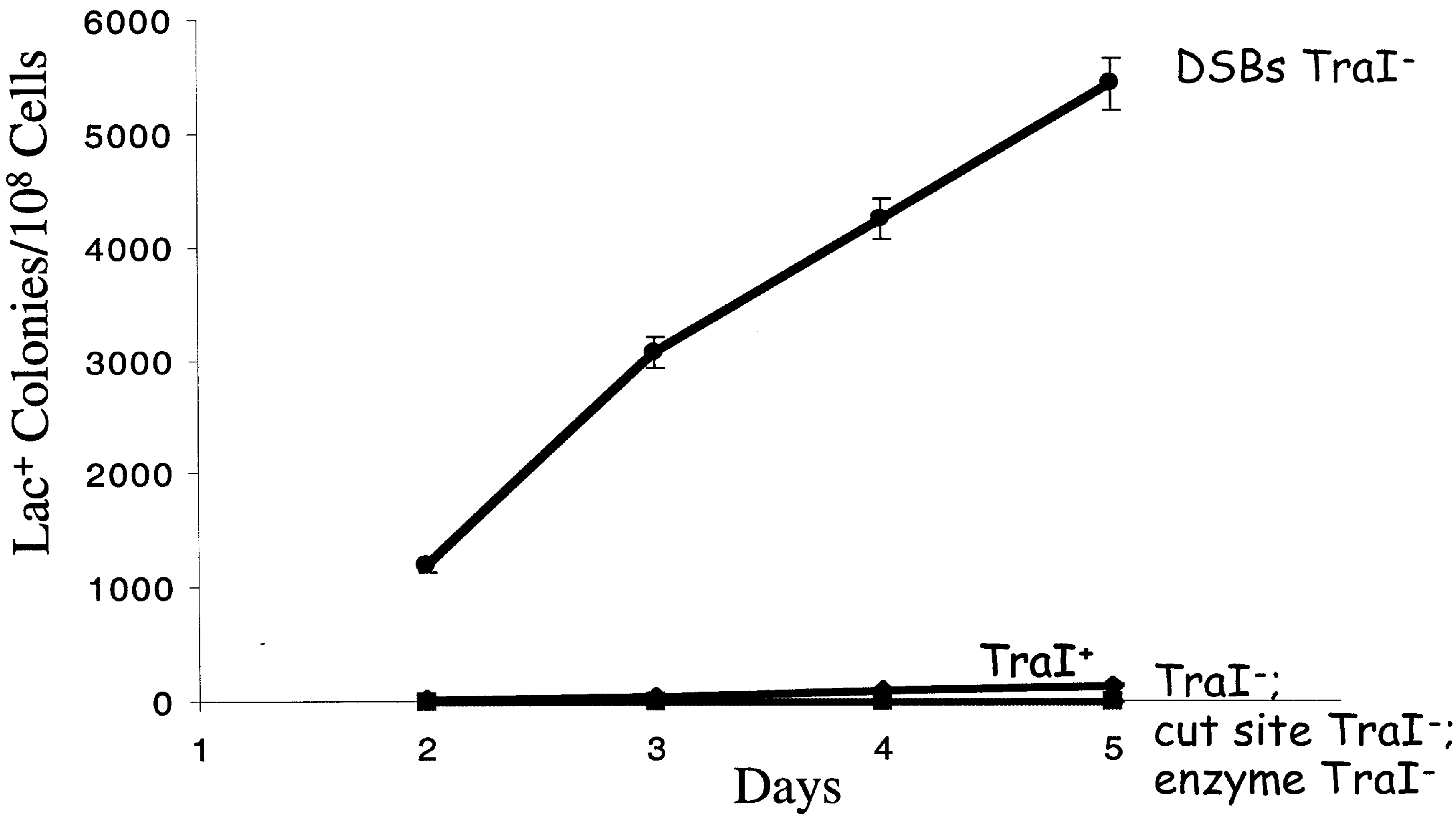

Figure 2

DSB to the right of *lac* ↑↑ mutation

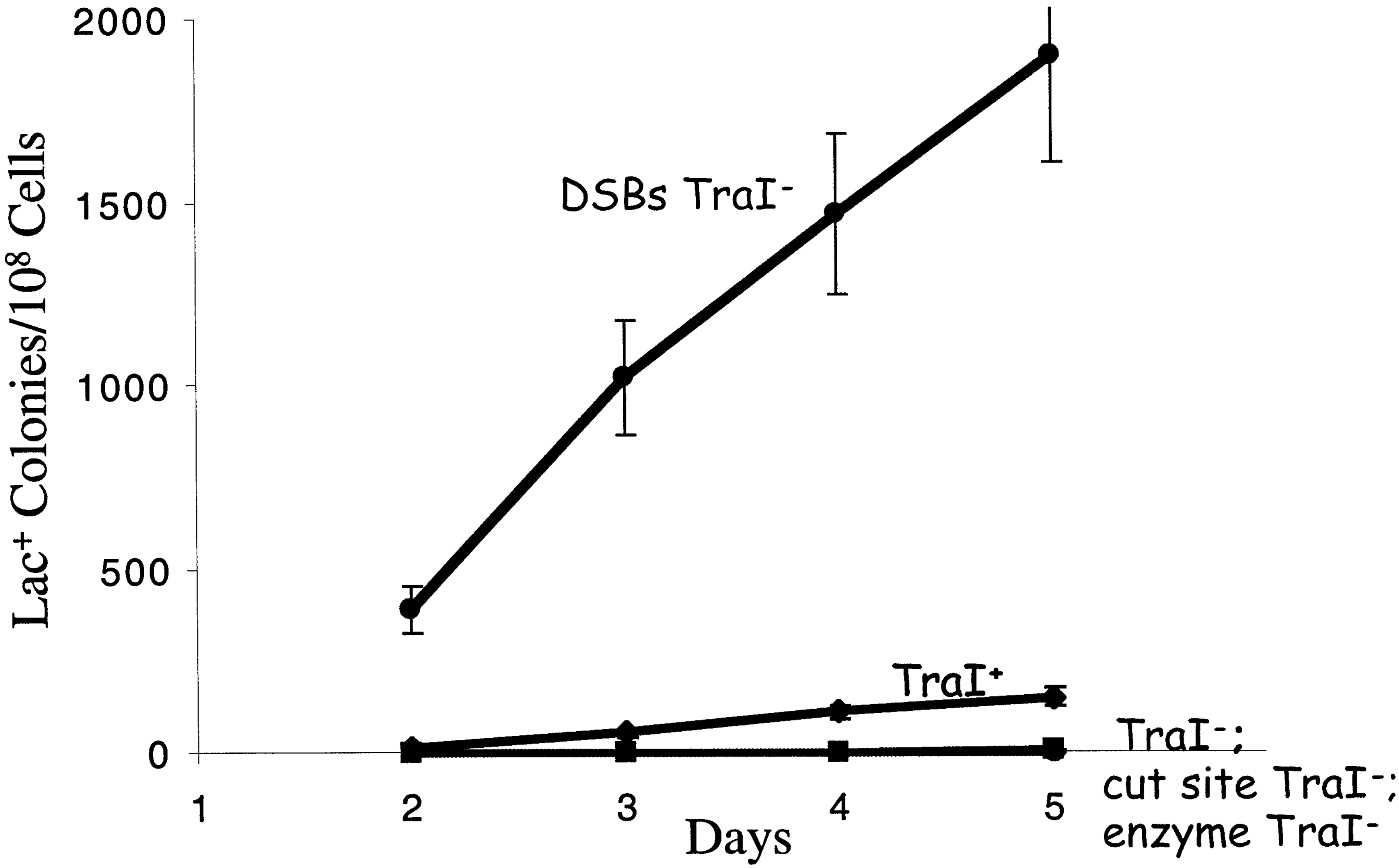

Figure 3

DSBs near *lac* ↑↑ adaptive amplification and point mutation

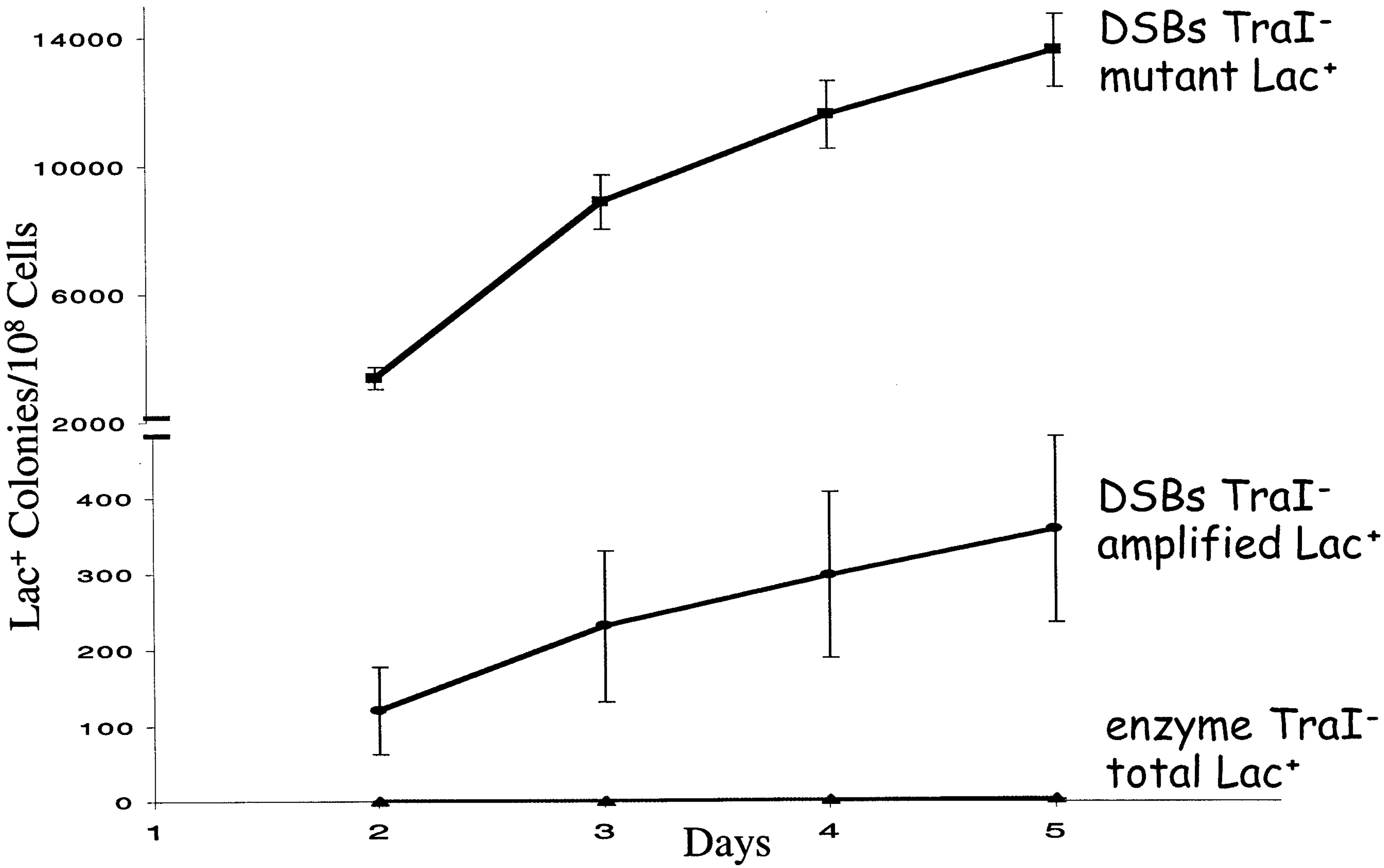

Figure 4

DSB-induced mutation requires Rec/Ruv proteins

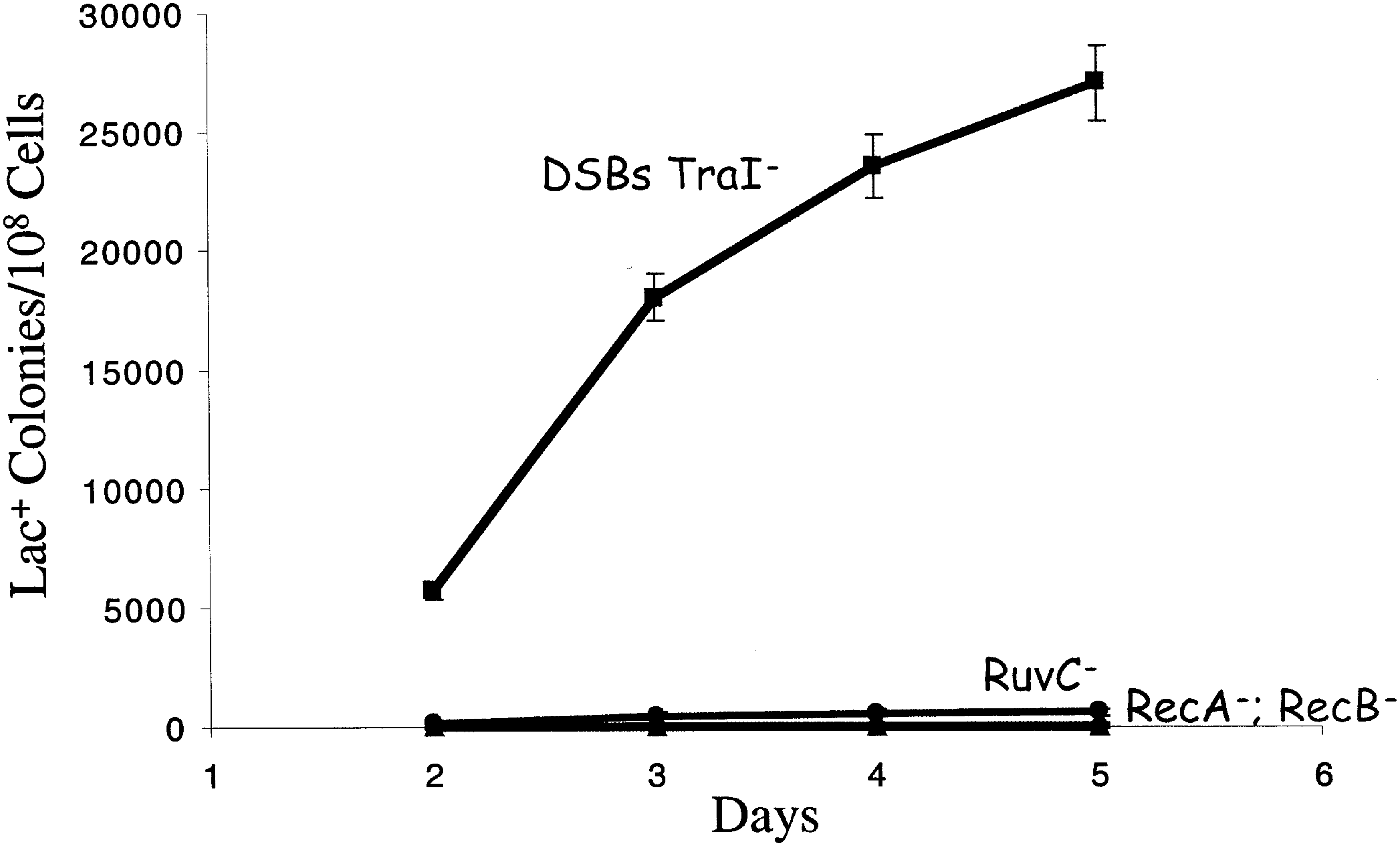

Figure 5

DSB-induced mutation requires DNA pol IV

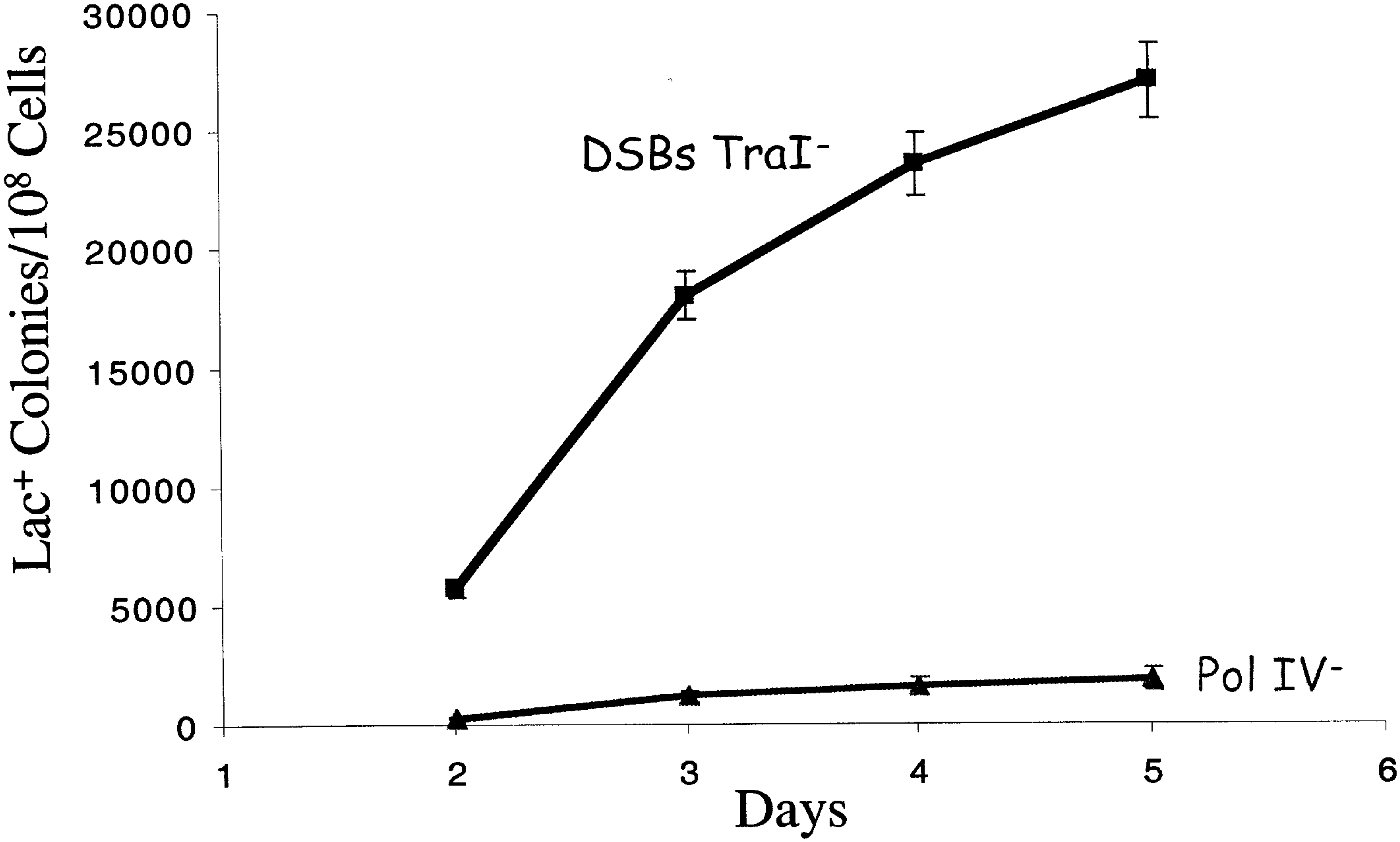

Figure 6

DSBs near *lac* ⤫ pol IV-dependent mutation during growth

TraI$^-$ DSBs

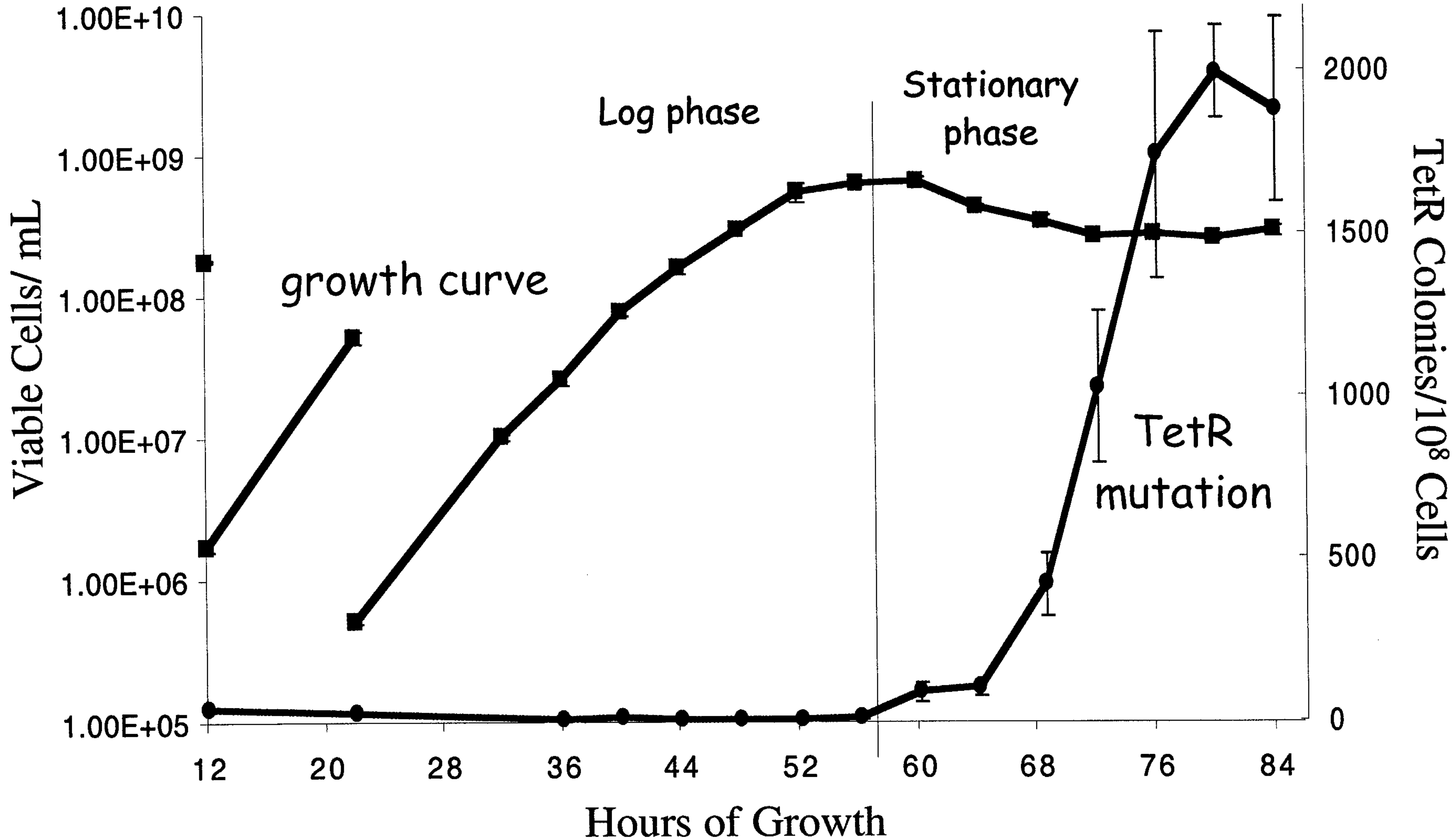

Figure 7

DSB-induced mutation requires RpoS

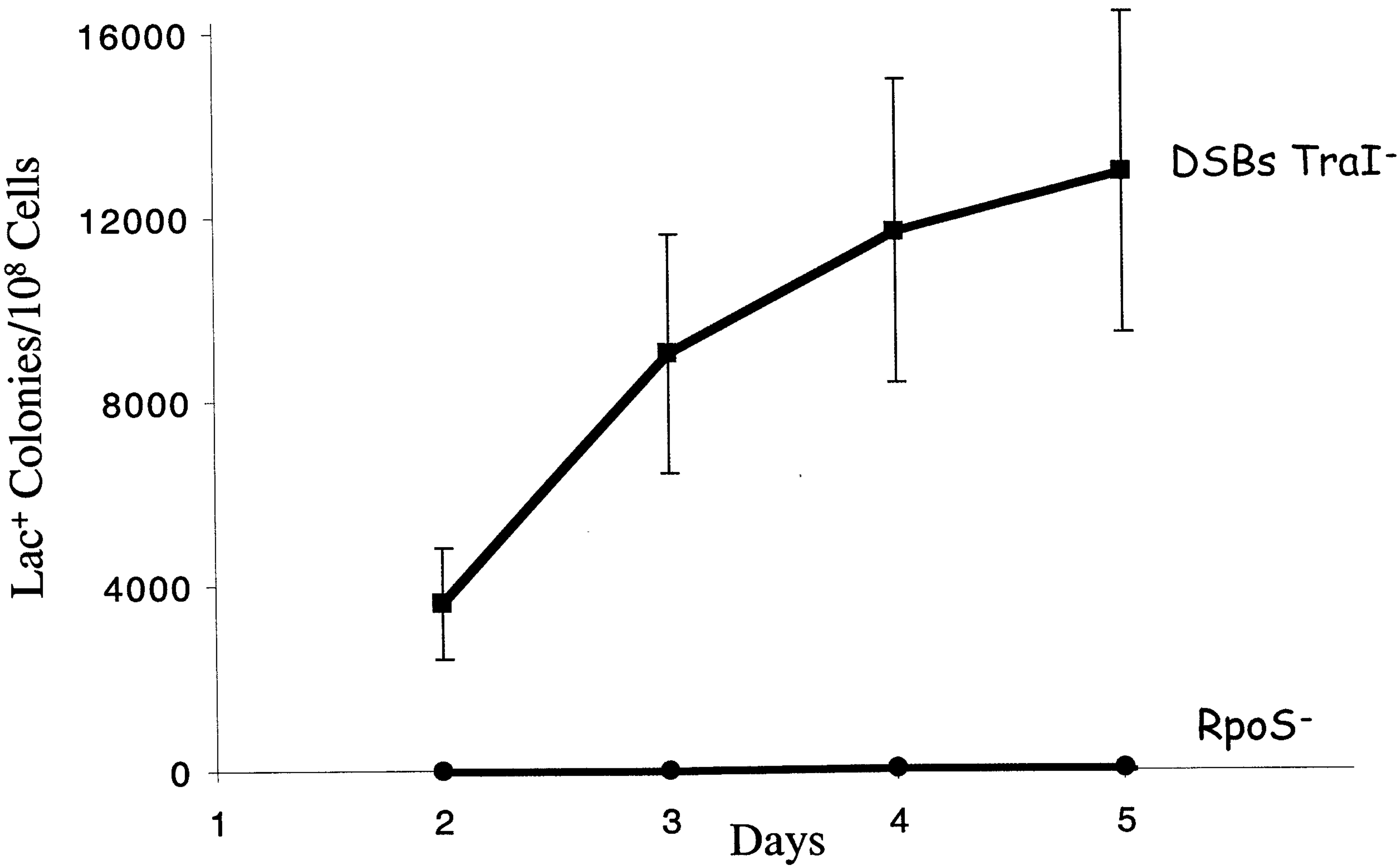

Figure 8

DSBs in *trans* to *lac* → mutation only slightly

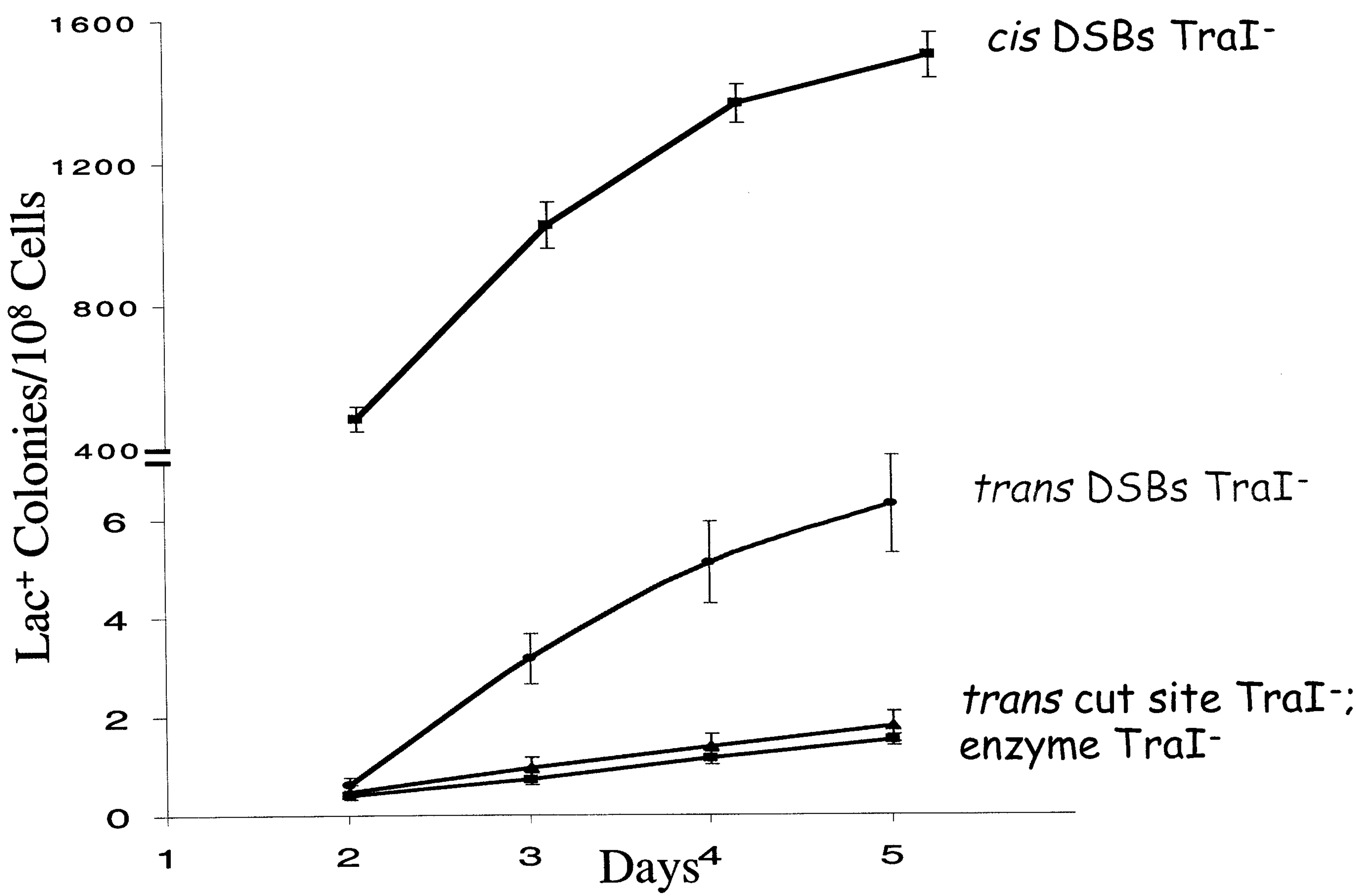

Figure 9

Homologous interactions with DNA ends ↑↑ mutation on the uncut molecule